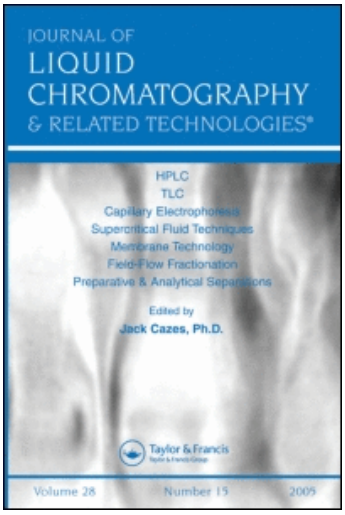

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A NEW CHEMICALLY DEACTIVATED SILICA-BASED REVERSE PHASE/ION EXCHANGE SUPPORT

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ABSTRACT

A new base-deactivated reverse phase chromatographic medium is described. Basic ligands have been incorporated into the silica matrix to interact with the free silanol groups, to supress their activity. Depending on the mobile phase, the support exhibits hydrophobic and/or ion exchange behavior and is stable between pH 1.5 - 7.5. Basic, acidic, polar, or apolar analytes can be separated with excellent selectivity and peak shape. Different selectivities were observed when the pH of the mobile phase was varied above and below the pK_a's of acidic analytes. Good separations were also observed for basic compounds, under chromatographic conditions where the bases were free or protonated, respectively.

INTRODUCTION

Silica-based reverse phase chromatographic media permit the separation of many organic compounds with reduced plate heights of 2 and asymmetries of 1.0. Other analytes, mainly strong organic bases and polar compounds, exhibit excessive tailing that is related to the interaction of these compounds with the

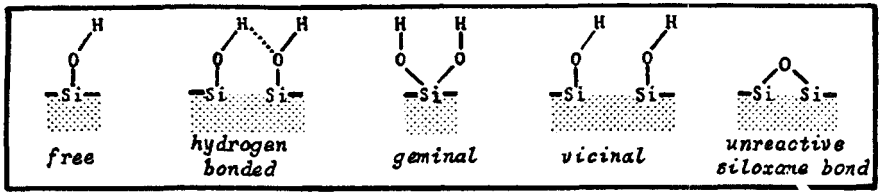


Figure 1. Configurations of the surface silanol groups.

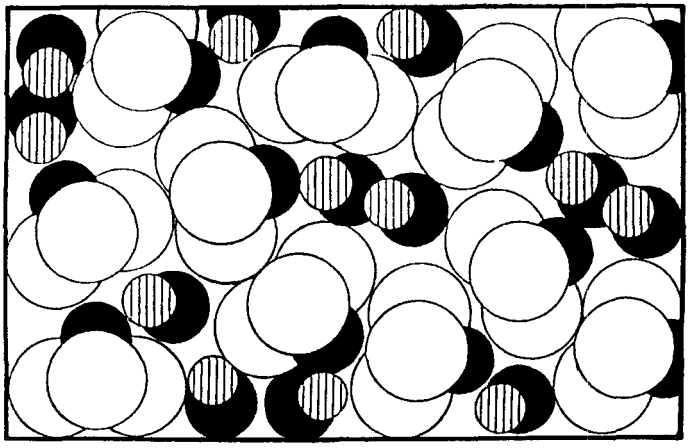


Figure 2. Silica surface with maximum coverage of trimethylsilyl (TMS) molecules. Circles represent projection of the TMS-methyl groups (open), silanol hydrogen atoms (hatched), and oxygen atoms of both (dark).

free silanols on the surface of the silica. To overcome this problem, a new base-deactivated reverse phase medium that minimizes the activity of the residual silanol groups was developed. On a hydrated silica surface, there are about 4.8 silanol groups/nm^2 or 8 μmole/m^2. Most of these silanols are hydrogen bonded to neighboring silanol groups, as shown in Figure 1. Other silanols are free as a result of unfavorable spatial configurations that prevent the formation of such bonds. These *free silanol* groups are more acidic and tend to associate more strongly with silanophilic analytes than do *hydrogen bonded* or self-associated silanols.

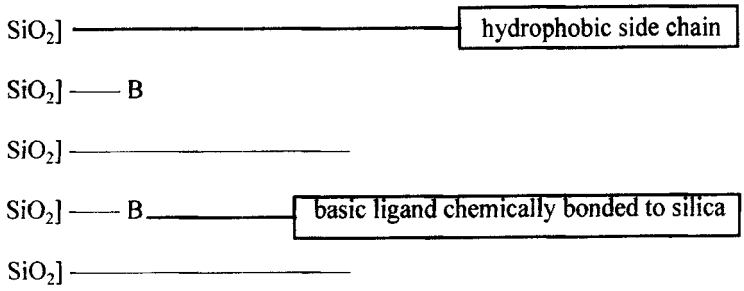
Reverse phase, silica-based, supports are synthesized by substituting the —OH of the silanol groups with dimethylalkylsilyl moieties to yield a brush-type reverse phase. The smallest of these moieties, trimethylsilyl, binds to the

surface at the 4 μmole/m^2 level, while the larger C_{18} homolog binds at the 3.2 μmole/m^2 level. This leaves about half of the 8 μmole/m^2 of the available silanol groups unchanged.[1]

It should be noted, also, that the population of **free** *silanols* on the surface of silylated silica increases as some of the remaining silanols (which were hydrogen-bonded before silylation) become more isolated and prevented from forming intra-hydrogen bonds (Fig. 2).

In reverse phase supports introduced a few years ago, bulky diisopropylalkylsilyl, instead of dimethylalkylsilyl moieties, were substituted for the —OH silanol-groups in an attempt to minimize band broadening and peak tailing, as well as increase the chemical stability of the bonded ligands.[2,3] However, though the diisopropylalkylsilyl ligands blocked access to some free silanols, others were still accessible, interacting with silanophilic analytes and resulting in broadened bands and tailing peaks for these compounds. A different approach to minimize the reactivity of the residual silanols in reverse phase supports was recently described.[4] In this approach, basic/polar ligands were chemically incorporated into the silica matrix. This approach was used to synthesize the ProTec-C_8 medium. Here, amine-type ligands were incorporated into the silica matrix of a C_8 bonded phase, resulting in a reverse phase support with ion-exchange character as shown in Scheme 1. These ligands form hydrogen bonds and ion pairs with the free silanols, internally suppressing (deactivating) their activity and enhancing the chromatography of basic, acidic, and polar analyses.

Scheme 1



In the presence of buffers between pH 2 - 7, the bonded basic ligands (—B) are in their conjugated acid form (—BH^+A^-, where A^- represents the buffer counter ion held on the stationary phase). When aqueous salts have no

buffer capacity, i.e., $NaClO_4$ at pH 7.0, the basic ligands stay predominantly free with only a small fraction protonated (—BH^+OH^-), causing a sharp *local* pH gradient between the basic surface and the bulk of the mobile phase.

As we shall later show, the ionic strength of the buffer is important for efficient and reproducible separations.

EXPERIMENTAL

Instruments

The chromatographic system consisted of a Model 300 pump (Gynkotek, Germerine bei Munchen, Germany); a Model 7125 Rheodyne injector fitted with a 20μL loop; a Spectroflow 759A Detector (Applied Biosystems, Mountain View, CA) and a ProTec-C_8, 150 mm x 4.6 mm column (ES Industries, Berlin, NJ).

Eluant flow rate was 1.0 mL/min and the eluate was monitored at 254 nm, except where otherwise indicated.

Chemicals

HPLC grade acetonitrile, methanol, and water were obtained from J. T. Baker (Phillipsburg, NJ). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI).

Sample Preparation

Samples were dissolved in mobile phase at concentrations such that detector signals were about 100 mV full scale.

RESULTS AND DISCUSSION

Column Conditioning Study

Because the ProTec series columns have, in part, ion exchange character, the ion exchange sites of the stationary phase are ion-paired with counter-ions

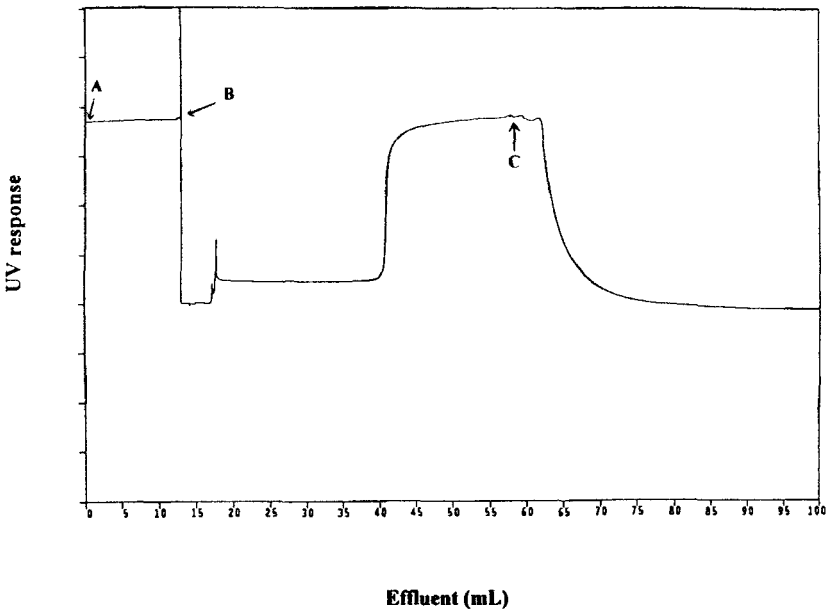


Figure 3. The breakthrough curve for triethylamine displacing phosphate ions. Column initially conditioned with 20:80 (v/v) methanol-50 mM K_2HPO_4, pH 7.0 at 1.0 mL/min until a steady baseline was obtained. Detector: 220 nm. A: Column bypassed and system washed with 80:20:0.2 (v/v) acetonitrile-water-triethylamine mobile phase. B: Column and system washed with triethylamine-containing mobile-phase. C: start column and system wash with 80:20 acetonitrile-water mobile phase.

of the buffer. When a new mobile phase buffer is used, the column must be reconditioned to replace the counter ions from the previous mobile phase buffer that were ion-paired to the basic ligands with counter-ions from the new buffer.

A series of experiments was performed to determine the amount of phosphate and trifluoroacetate (from trifluoroacetic acid) bound to the column. For each experiment, the column and system were washed to assure that only phosphate, triethylamine, or trifluoroacetate were breaking through. The chromatograms shown in Figures 3 and 4, respectively, show that about 400 μmole of triethylamine (TEA, equivalent to about the same quantity of phosphate, assuming a 1:1 triethylamine-phosphate ion-pair) were required to release the phosphate ions and about 1040 μmole of trifluoroacetate ions were ion paired to the protonated basic ligands.

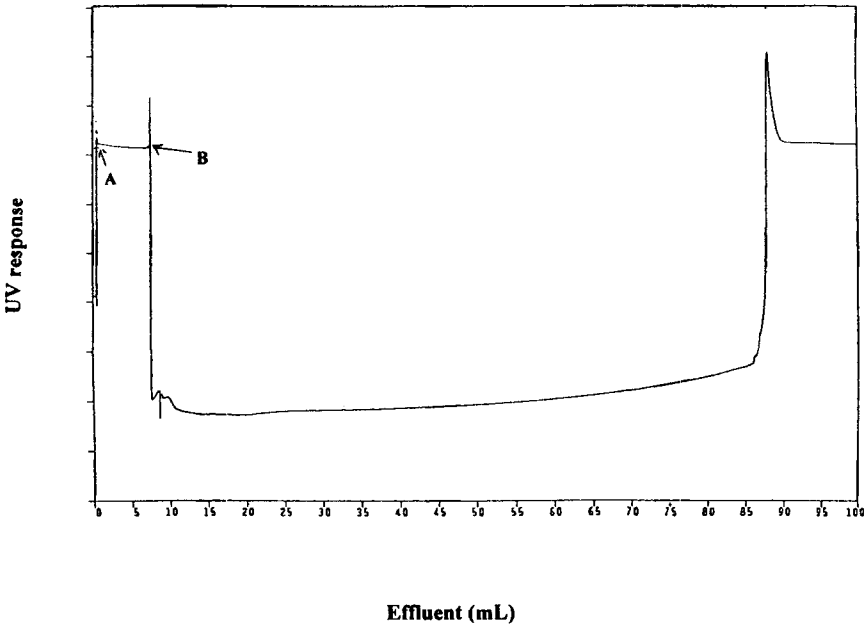


Figure 4. Trifluoroacetic acid breakthrough curve. Detector: 220 nm; Column washed with an 80:20 (v/v) acetonitrile-water mobile phase until a steady baseline was obtained. A: column bypassed and system washed with a 40:60:0.1 (v/v) acetonitrile-water-trifluoroacetic acid mobile phase. B: column and system washed with trifluoroacetic acid-containing mobile phase.

The reverse phase and ion-exchange behavior of the ProTec-C_8 column for the separation of basic, acidic and polar and apolar compounds was characterized in a series of experiments. As shown in Figure 5A, strong organic bases, such as cyclobenzaprine and amitriptyline, were separated as free bases with good resolution and peak shape using a mobile phase consisting of 50:50 (v/v) acetonitrile-50 mM $NaClO_4$.

Both compounds, which were injected as their hydrochloride salts, were converted, on column, by ion-exchange, into free bases by the basic ligands that are an integral part of the ProTec-C_8 medium. The better resolution of these compounds as free bases is due, in part, to the enhanced hydrophobic partitioning of the free bases compared to that of their conjugated acid forms in a buffered pH 7.0 mobile phase, as shown in Figure 5B.

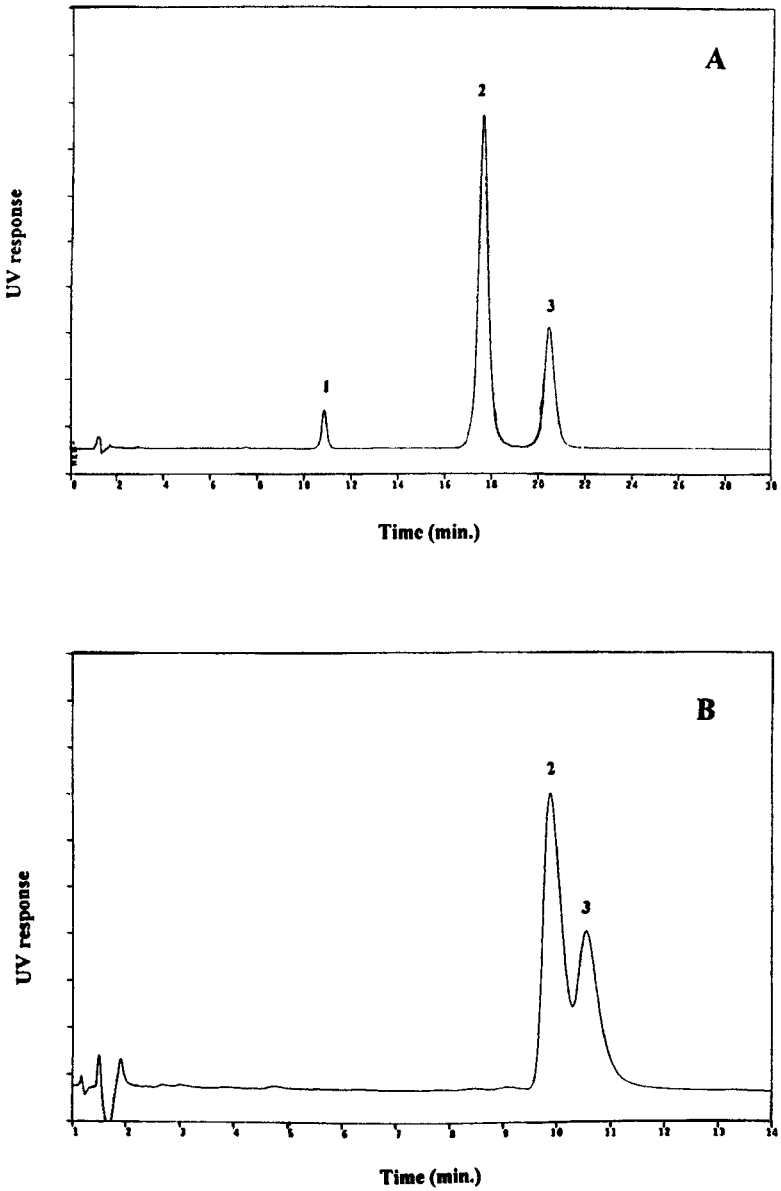


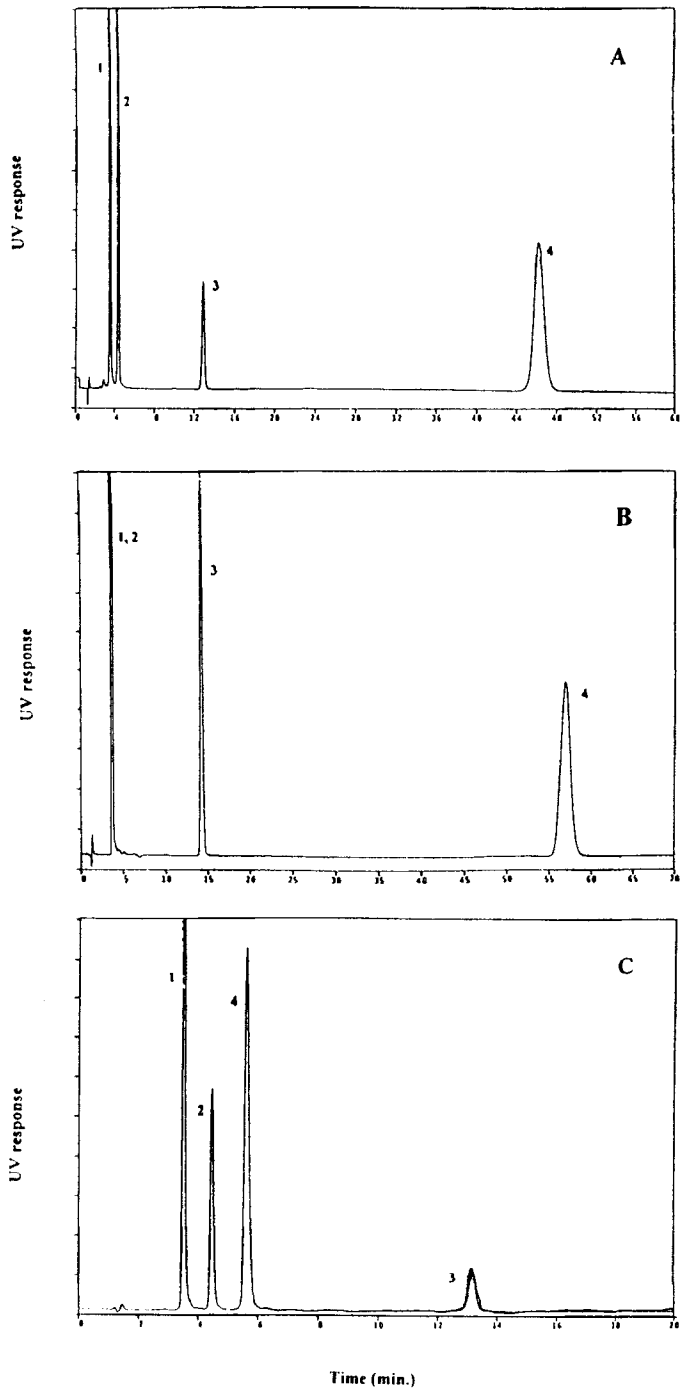
Figure 5. Separation of free bases and their conjugated acid forms in neutral unbuffered and buffered mobile phases. Mobile phase A: 50:50 (v/v) acetonitrile-50 mM $NaClO_4$; mobile phase B: 40:60 (v/v) acetonitrile-50 mM K_2HPO_4, pH 7.0. 1 = naphthalene (internal standard), 2 = cyclobenzaprine, 3 = amitryptyline.

Since the free basic ligands are gradually converted to their conjugated-acid form by ion exchanging the hydrogen chloride from the basic analytes, the column should be periodically washed with a 0.02% TEA-containing mobile phase to remove the accumulated HCl. In addition, when aqueous mobile phases without buffer capacity are used, the dissolved carbonic acid from ambient air accumulates on the column and slowly decreases the retention times of cationic compounds (results not shown). In this study, an amino guard column was placed before the injector to remove dissolved carbonic acid when $NaClO_4$-containing mobile phases were used.

Since the basic ligands bonded to silica matrix suppress the activity of the free silanols, it is expected that triethylamine will control only pH and not further suppress the silanophilic activity of these free silanols, and the separation will depend on hydrophobic interactions between the analyte, support, and mobile phase. To illustrate this, a mixture of typical weak and strong bases and polar and apolar compounds was separated using unbuffered, buffered pH 7.0, and basic mobile phases. The four test compounds: aniline (PK_a 4.63),[5] amitriptyline (pK_a 9.4),[6] phenol (pK_a 9.95),[7] and toluene (used as an indicator of chromatographic performance because it does not exhibit band broadening due to interactions with free silanols) were separated using a 40:60 (v/v) acetonitrile-water mobile phase on a new column that was not exposed to a buffer solution as shown in Figure 6A. Separations similar to that shown in Figure 6A were also obtained with a mobile phase of 40:60 (v/v) acetonitrile-water on a column that was previously exposed to different buffers and pre-washed with a TEA-containing mobile phase (results not shown). Using the high pH TEA-containing mobile phase, phenol is converted to its phenolate form and co-elutes with aniline as shown in Figure 6B.

Under the conditions used in Figures 6A and 6B, the basic analytes, aniline and amitriptyline, elute as free bases. In the presence of a buffer at pH 7.0, aniline is a free base and its retention is unaffected by a change in the conditions of the mobile phase, as shown in Figure 6A-C. On the other hand, in the presence of a pH 7.0 buffer, amitriptyline is protonated and the 10-fold difference in retention times using the basic and buffered pH 7.0 mobile phases is a consequence of the greater retentivity of the free base, as shown in Figures 6A and 6C, respectively.

Figure 6 (right). Chromatograms of weak and strong base and polar and apolar compound test mixture. Mobile phase A: 40:60 (v/v) acetonitrile-water; mobile phase B:40:60:0.02 (v/v) acetonitrile-water-triethylamine; mobile phase C: 40:60 (v/v) acetonitrile 50 mM K_2HPO_4 /KH_2PO_4, pH 7.0. 1 = aniline, 2 = phenol, 3 = toluene, 4 = amitriptyline.



Effect of Ionic Strength

In the presence of a buffer at pH 7.0, most of the basic ligands bonded to the medium are protonated. A rapid decrease in the ionic strength of the buffer, KH_2PO_4/K_2HPO_4 (pH 7.0), from 50 mM to 2 mM, causes the positively charged amitriptyline to elute close to the void volume (k' ~0.7) in an ion-exclusion mechanism caused by the repulsion between the positively charged analyte and the positively charged ligands under low ionic strength conditions.[8,9] The retention times of the neutral analytes (aniline, phenol and toluene) are not affected.

After extensively washing the column with the 2 mM buffer, the retention time of amitriptyline gradually increased because of a gradual decrease in the charge density on the surface of the medium (results not shown). This behavior illustrates how decreasing the ionic strength of the buffer can be useful in enhancing selectivity by changing the chromatographic behavior of interfering protonated analytes.

Using acidic mobile phases (0.1% TFA), organic acids and phenolic compounds are efficiently separated. At low pH (pH~1.5), the common organic acids are protonated and are retained on the column, mostly due to hydrophobic interactions with the stationary phase. They elute in symmetrical bands with the later eluting compounds showing better peak symmetry (see Fig. 7A). Salicylic acid, which is a stronger acid than the regular carboxylic acids, has an unusually long retention time compared to its retention on other base-deactivated reverse phase columns due to mixed ion-exchange/ hydrophobic interactions [k' = 5.6 with a 30:70:0.1 (v/v) AcCN:H_2O:TFA].

The ion-exchange sites of the ProTec-C_8 column bind carboxylate groups of organic acids and 'prevent' their elution by low ionic-strength buffers. As a result mobile phases containing buffers at pH's above the pK_a's of organic acids will produce substantially longer retention times than on conventional reverse phase columns. Using a 30/70 (v/v) acetonitrile-50 mM ammonium acetate mobile phase (adjusted to pH 5.0 with NH_4OH) resulted in very poor peak shapes and long retention times, i.e., benzoic acid eluted after 60 minutes (results not shown).

When the ammonium acetate concentration was increased to 200 mM, the separation shown in Fig. 7B was obtained. Thus, when separating a mixture containing organic acids, after the separation of the basic, polar or nonpolar compounds is achieved, an ionic-strength gradient can be used to elute/separate acids in a mobile phase with the same organic solvent strength.

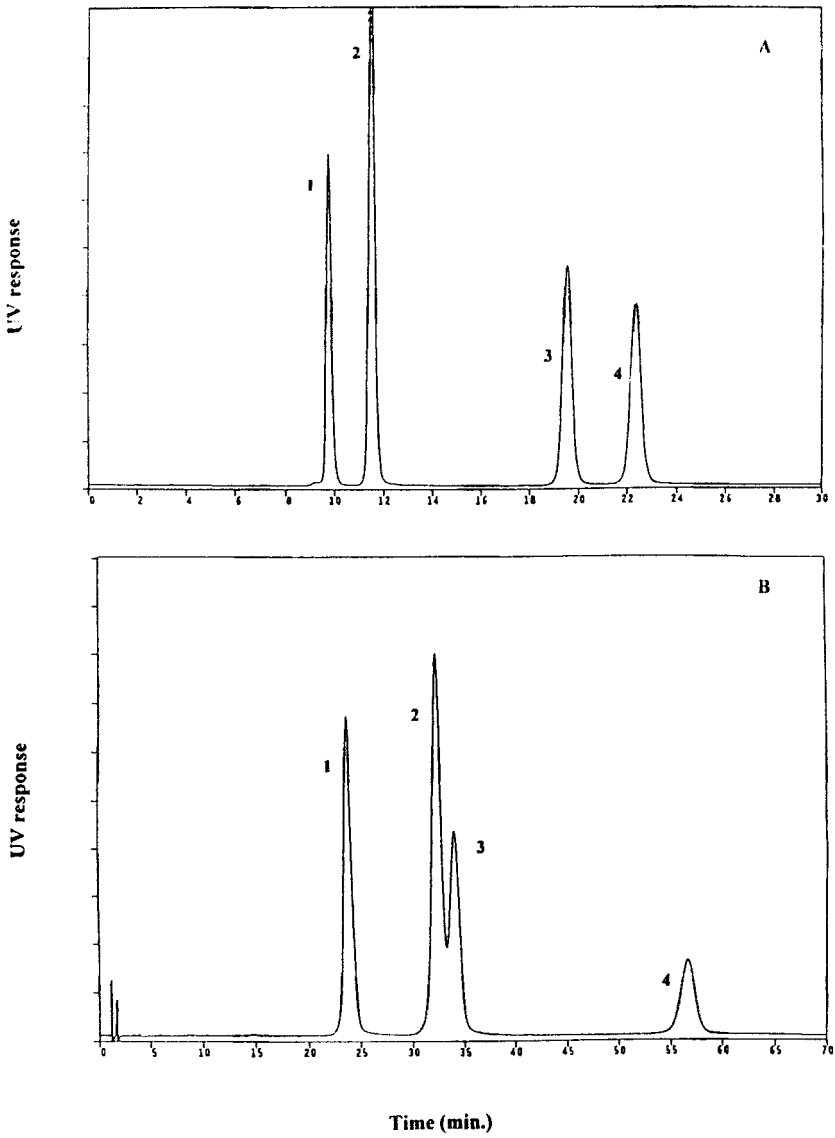


Figure 7. Separation of organic acids below and above their pK_a values. Mobile phase A: 20:80:0.1 (v/v) acetonitrile-water-trifluoroacetic acid; mobile phase B: 30:70 (v/v) acetonitrile-water containing 200 mM ammonium acetate and adjusted to pH 5.0 with NH_4OH. 1 = phenylacetic acid, 2 = benzoic acid. 3 = o-toluic acid, 4 = p-toluic acid.

Table 1

Retention Times, Plate Numbers and Peak Asymmetries for Toluene and Naphthalene for Column Stability Study

	Toluene			Naphthalene		
Injection Time (h)	R_t* (min)	Number of Plates	Asymmetry	R_t* (min)	Number of Plates	Asymmetry
50:50:0.1 (v/v) Acetonitrile-Water-Trifluoroacetic Acid at 30°C:						
1	5.76	14,300	1.25	7.40	15,400	1.10
17	5.63	14,400	0.99	7.27	15,200	1.20
23	5.64	14,400	1.08	7.27	15,200	0.94
40	5.68	15,500	1.21	7.36	16,000	1.26
66	5.72	14,100	0.98	7.43	14,800	0.94
40:60:0.1 (v/v) Water-Methanol-Trifluoroacetic Acid at 30°C:						
17	6.65	12,200	1.14	10.24	12,700	1.07
47	6.70	12,200	1.11	10.35	12,700	1.01
63	6.75	11,500	1.08	10.48	12,000	0.97
50:50:0.02 (v/v) Acetonitrile-Water-Triethylamine at 30°C:						
1	6.88	15,700	0.98	9.48	16,000	1.04
18	6.92	14,800	1.14	9.64	15,300	0.86
65	7.03	12,600	0.83	9.93	12,100	0.75
87	7.07	12,200	0.80	10.02	11,900	0.77

* Column washed between injections at 0.3 mL/min. Flow rate for each injection was 1.0 mL/min.

Stability of the ProTec-C_8 Medium

The particular chemistry utilized for the synthesis of the ProTec-C_8 medium allows the use of mobile phases containing 0.1% (13 mM) trifluoroacetic acid or other strong acids such as methanesulfonic acid of similar molarities. The results presented in Table 1 show that, after long exposure to mobile phases containing strong organic acids, separation efficiency and peak asymmetry are relatively unchanged, indicating good stability of the support. After long use with a basic mobile phase, i.e., 50:50:0.02 (v/v)

acetonitrile:water:TEA at 30°C, retention times increase and the number of theoretical plates and peak symmetry decrease. This indicates that the silica matrix slowly dissolves, but the ligand-silica surface bond is stable.

CONCLUSIONS

The new base-deactivated ProTec-C_8 medium incorporates basic groups in the bonded phase to internally suppress the activity of the free silanol groups. Using buffers at pH 7 0, the bonded ligands are in their cationic form and strong organic bases migrate through the column in their ionic forms. In buffers whose ionic strength is greater than 20 mM, the charge density of the anionic counter ions suppresses the coulombic repulsion between the positively charged analytes and basic ligands, resulting in hydrophobic retention of the analytes. When the counter ion concentration is decreased — when the ionic strength is decreased from 50 mM to 2 mM — the resulting increase in repulsive force decreases the retention of positively charged analyses. This can be useful for the resolution between positively charged and neutral analytes that were similarly retained using the higher buffer concentration. On the other hand, at pH 5.0, acidic analytes, for example, migrate through the column as carboxylate ions and bind to the basic ligands. As a result, their separation is enhanced with the use of an ionic strength concentration gradient.

Conditions where the basic groups of the ProTec-C_8 medium is free permit the on-column conversion of strongly basic analytes to their free base form. Separation of these free bases allows greater selectivity than was obtained for the separation of the same bases in their conjugated-acid form. Moreover, the stability of the medium at $pH_{app.}$ 1.5 allows acidic analytes to be separated under conditions in which their dissociation is suppressed.

The chromatographic characteristics of the ProTec-C_8 medium provides the analyst with a flexible chromatographic tool that can be used over a wide pH range for the enhanced separation of acids, bases and polar analytes. This unique medium allows for an ion-exchange contribution to reverse phase chromatography to enhance separation and selectivity.

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